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Loss of the retinoblastoma tumor suppressor protein (pRb) can contribute to breast tumor formation. However, many breast tumors retain expression of normal pRb, indicating that other genetic events may interfere with pRb's function. For example, overexpression of cyclin D1 can lead to constitutive inactivation of pRb through phosphorylation. We have demonstrated that cyclin D1 in oncogenic in cultured cells, and are using this assay to define regions and functions of cyclin D1 needed for transformation. Interestingly, a previously defined pRb-interaction domain in cyclin D1 is dispensable for phosphorylation and transformation, and a mutant of cyclin D1 incapable of kinase activation retains partial ability to inactivate pRb. These data suggest that cyclin D1 may have a unique method of circumventing pRb function that is independent of physical association with pRb and phosphorylation. We are creating animal models expressing cyclin D1 mutants that will allow a true in vivo determination of cyclin D1 functional domains in breast development and cancer. We have also produced and characterized a temperature sensitive pRb that is being used to identify gene targets of pRb. This mutant has revealed a cell-death effect resulting from pRb loss that may be due to the establishment of a pRb-directed, terminal cell cycle exit consistent with senescence.

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INTRODUCTION

Breast cancer, like other cancers, results from the hyperactivity of growth-promoting oncoproteins and the loss of growth suppressing proteins (tumor suppressors). Many oncoproteins and several tumor suppressor proteins have been identified in recent years. Among the most commonly altered tumor suppressor proteins is the retinoblastoma protein, pRb. pRb function is lost in all retinoblastoma tumors, where it can lead to hereditary cancer, but is also involved in a variety of other tumors due to somatic inactivation. Reintroduction of the RB-1 cDNA into such cells inhibits their proliferation, supporting the role of pRb-inactivating mutations in formation of neoplastic cells (reviewed in Weinberg, 1991). This tumor-suppressive property of pRb is believed to result from pRb's ability to regulate progression through the cell cycle.

A central role for pRb in the control of cellular proliferation is also suggested by the observation that pRb is targeted by the oncoprotein products of several DNA tumor viruses. This interaction presumably serves to inactivate the growth-suppressive properties of pRb in infected or transformed cells. One mechanism by which oncoprotein-mediated inactivation may be achieved is through the dissociation of protein complexes between pRb and growth promoting molecules. For example, pRb has been reported to associate with the transcription factor E2F, which may be involved in the regulation of many genes required for DNA synthesis. The association of pRb with E2F may prevent the activation of these genes until the G1/S boundary, at which time the pRb/E2F complex dissociates, resulting in the release and activation of E2F. Because association of pRb with E2F seems to involve the same region of pRb (the "pocket") that is required for association with the viral oncoproteins, the binding of viral oncoproteins to pRb may release and activate E2F, resulting in the removal of a block to progression into S phase (reviewed in Tiemann, et al., 1997).

As is the case with a variety of human tumor types, some thirty percent of breast tumors show loss of pRb expression (Weinberg, 1991); however, other tumors have apparently wild-type pRb, and may have suffered alterations in one or another cellular proteins which interact with pRb. This may in turn lead to constitutive inactivation or circumvention of pRb function. A clue to the identity of such regulators of pRb is given by the fact that pRb is normally controlled by phosphorylation mediated by cyclin-dependent kinases (cdks; Lin, et al., 1991; Lees, et al., 1991). These cdks are controlled in turn by cyclins, regulatory subunits which lead to cyclic activity of their partner kinases. Work from many laboratories suggests that D-type cyclins in combination with cdk4 or cdk6 can initiate pRb phosphorylation in G1 and cyclin E/cdk2 complexes may continue or expand on this phosphorylation just prior to S phase entry (Ewen, et al., 1993; Kato, et al., 1993). Indeed it has been suggested that both cdk4 and cdk2 activity may collaborate to fully inactivate pRb prior to S phase entry (Hatakeyama, et al., 1994). The activity of these cyclin/cdk complexes is further regulated by positive and negative phosphorylation of the cdk subunit. In addition, several proteins have recently been identified that serve to stoichiometrically inhibit the function of cyclin/cdk complexes (reviewed in Morgan, 1995). Thus, these cyclin-dependent kinase inhibitors, or CKIs, together with cdk-modifying enzymes and cyclins

represent potential targets for oncogenic mutations that may lead to deregulated cell cycle progression.

Importantly, cyclin D1 has been shown to be overexpressed in approximately fifty percent of breast tumors (Lammie, et al., 1991; Schuuring, et al., 1992; Buckley, et al., 1993; Keyomarsi and Pardee, 1993), as well as in cancers of the parathyroid, blood and squamous epithelium (Motokura, et al., 1991; Rosenberg, et al., 1991a,b; Withers, et al., 1991). Thus, deregulated D-type cyclin expression may be oncogenic, leading to aberrant cellular proliferation perhaps by interfering with the function of pRb. We have shown that cyclin D1 can indeed act as an oncogene, cooperating to transform cultured cells in cooperation with a mutant adenovirus E1A oncoprotein which has lost the wild-type capacity to bind and inactivate pRb (Hinds, et al., 1994), and transgenic mice engineered to overexpress cyclin D1 in the breast demonstrate aspects of tumorigenesis (Wang, et al., 1994).

In a conceptually similar manner, loss of CKI function may also lead to loss of pRb function in pRb-positive tumors. p16INK4a, an inhibitor tailored specifically to prevent the function of cdk4 and cdk6, is deleted or mutated in many cancers, presumably leading to hyperactivity of the cyclin D/cdk4(6) complex that initiates pRb phosphorylation (reviewed in Weinberg, 1995). p16 is thought to act as a direct competitor of D-type cyclins for cdk4/6 association, preventing the activation of these kinases when conditions are inappropriate for cellular proliferation. Consistent with a role for cdk4 as a target of the tumor-suppressive effects of p16INK4a, cdk4 has been found to be amplified in a number of tumor types, most notably in glioblastomas (Schmidt et al., 1994; He et al., 1994; Ichimura et al., 1996). In addition, a mutant form of cdk4 that cannot bind p16INK4a has been found in melanoma cells (Wölfel, et al., 1995) . Similarly, we have recently shown that cdk6 is specifically activated in oral epithelial tumors through gene amplification, suggesting a direct role in the genesis of these tumors (Timmermann, et al., 1997). Thus, it is clear that tumor cells exploit at least four mechanisms (Figure 1) to abrogate pRb function: elimination of pRb itself, elimination of the negative regulator p16, and overexpression of cyclin D1 or cdk4/6.

The molecular events outlined in Figure 1 may be oncogenic solely due to inactivation of pRb, and as such provide several alternatives to RB alteration in cancer. In favor of this "phenotypic identity" among mutations in the RB pathway is the fact that such mutations are usually mutually exclusive. That is, breast tumors are found to contain either pRb loss or cyclin D1 overexpression, but not both. Similarly, overexpression of cdk4 and loss of p16INK4a are mutually exclusive events in glioblastoma. However, differences in the frequency of mutation of each pathway member occur in distinct tumor types, suggesting that alterations in cyclin D/cdk4 activity may be more profound than loss of pRb in some cell types. For example, as stated above, cyclin D1 amplification is highly favored over pRb loss in breast tumors. Further, p16INK4a loss is nearly absolute in melanoma, and cdk6 activation appears to occur in all oral epithelial tumor cell lines. These results suggest that while pRb inactivation may be one important outcome of such alterations, different cell types may select certain mutations due

to additional effects that favor proliferation in comparison to pRb loss alone. Recently, functional explanations for some of these asymmetries have been suggested. For example, loss of the p16INK4a locus removes not only the p16 cdk inhibitor, but also often inactivates a second gene encoding p19ARF, a protein that may mediate the tumor suppressive effects of p53 (Kamijo, et al., 1998; Pomerantz, et al., 1998; Zhang, et al., 1998). Thus, loss of two tumor suppressors may favor alteration of this locus in many tumor cells. In a conceptually similar way, consistent activation of cdk6 versus cdk4 in oral epithelial cells or overexpression of cyclin D1 in breast tumors may be the result of multiple tumor inducing functions of these single events. Indeed, it has been suggested that cyclin D1 can activate the transcriptional function of the estrogen receptor in a kinase and ligand independent manner, a function that could favor its overexpression in breast tumor cells (Neuman, et al., 1997; Zwijsen, et al., 1997). Our current focus is on understanding the mechanisms by which D-type cyclins and cdk4/6 can act as oncogenes, particularly in roles that extend beyond inactivation of pRb.

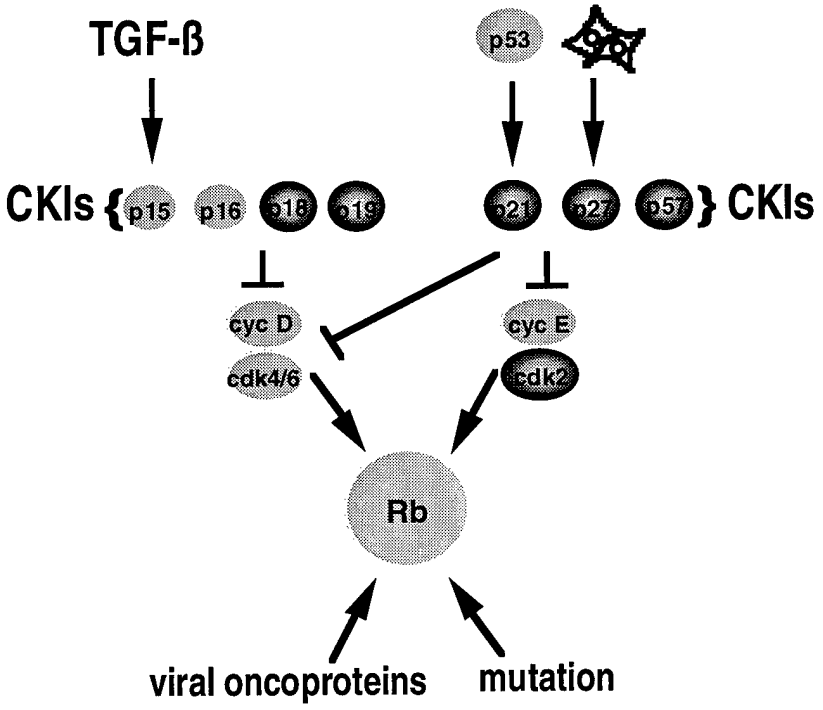

Figure 1. Interaction of cell cycle machinery, pRb and p53. Cyclin-dependent kinase (cdk) inhibitors (CKIs), cyclins and cdks involved in G1-S progression are shown. These proteins regulate the function of the Rb protein, a central target of oncogenic mutations. It has long been realized that such mutations inactivate pRb, and this can also be achieved by viral proteins, leading to uncontrolled proliferation. More recently, the cell cyclin components shown in gray have been demonstrated to be altered in tumors and/or function as oncogenes or suppressors in culture. Note that the CKIs are mediators of the growth suppressive influence of a variety of cell cycle inhibitors, such as TGF-ß, p53 and cell-cell contact. This pathway suggests that there are many ways to ultimately

deregulate pRb, and most tumor cells may contain alterations in one of these components.

We and others have shown that D-type cyclins can physically associate with pRb in a manner analogous to the viral oncoproteins (Ewen, et al., 1993; Kato, et al., 1993; Dowdy, et al., 1993), a property of cyclin D1 that may explain its prevalence as a target of oncogenic activation. Paradoxically, however, this direct pRb binding is dispensable for transformation (Hinds, et al., 1994), and, as shown below, for phosphorylation of pRb. Intriguingly, we have recently found that a mutant cyclin D1 protein (KE) which is altered in the cdk-binding region not only fails to transform primary cells, but is dominant over the wild-type protein in this capacity. Thus, cointroduction of mutant and wild-type cyclin D1 genes leads to no increase in transformation frequency, suggesting a dominant-negative function of the mutant protein. This mutant protein can be expressed in conjunction with wild-type E1A, however, and is thus not itself lethal to cells in the absence of overexpressed cyclin D1. More detailed understanding of the mechanisms behind the inhibition of cyclin function could lead to antiproliferative products using existing technologies and may be specific to those cells overexpressing certain cyclins, leaving normal proliferating cells relatively unaffected. The properties of D-type cyclins already uncovered in our prior research and those to be elucidated by the experiments described below provide an excellent opportunity for clinical intervention in aberrant cell cycle control, and could thus provide an important adjunct to the pharmaceutical treatment of human cancers. In addition, we seek to identify other upstream regulators or downstream targets of pRb that can inactivate pRb upon overexpression. Such candidate oncoproteins may be operative in the significant fraction of tumors that do not show direct inactivation of pRb. To address these issues, the following specific aims were originally proposed:

1) Use mutant pRb proteins that have been characterized for function as growth suppressors and substrates for the pRb kinase to determine if the association of D-type cyclins with pRb is required for pRb function or phosphorylation or both.

2) Identify a kinase activity precipitable with antibodies specific to a tagged D-type cyclin and use this functional assay to probe the effect of pRb binding on D-type cyclin function.

3) Introduce a dominant-negative D-type cyclin into cells transformed by wild-type D-type cyclins and control cells in effort to specifically prevent the proliferation of the transformed cells.

4) Identify other substances, e.g. antisense oligonucleotides or interfering peptides, which could cause cessation of proliferation of cyclin D-transformed cells in vitro and in vivo.

5) Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

RESULTS AND FUTURE DIRECTIONS

Specific aims 1 and 2: Mapping of pRb and cyclin D1 domains required for transformation.

Because catalytic partners of cyclin D1 were identified as cdk4 and cdk6 several years ago, it was unnecessary to develop the assay proposed in aim 2. Further, it was shown that the absence of pRb or its functional disruption by viral oncoproteins leads to a loss of cyclin D/cdk complexes, although the mechanism of this remains unclear (Bates, et al., 1994). We have therefore de-emphasized aim 2 and focus our efforts on cyclin D1 function in transformation on aim 1. In this case, despite a clear role for mediating cyclin D/pRb interaction in vitro (Dowdy, et al., 1993; Ewen, et al., 1993; Kato, et al., 1993), it is unclear what the role for the LXCXE domain is in vivo, since its disruption by point mutation often does not lead to functional inactivation (Dowdy, et al., 1993; Hinds, et al., 1994).

Thus, although it is clear that cyclin D1 can function as an oncogene and does so at least in part by interfering with the pRb protein, the exact mechanism by which this is achieved is unclear. For example, cyclin D1 may simply activate its cognate kinase partners, cdk4 and cdk6, to directly phosphorylate pRb. Alternatively, excess cyclin D1/cdk complexes may compete with or lead to inactivation of any of a variety of CKIs now known to regulate cdk activity. Finally, although the absence of pRb seems to negate the need for cyclin D1 in tumor cells (Lukas, et al., 1995; Parry, et al., 1995), cyclin D1/cdk complexes may have other cellular targets. Examples of such targets are the pRb homologues p107 and p130 (Beijersbergen, et al., 1995). These proteins are homologous to pRb in the region used to associate with viral oncoproteins. Indeed, these proteins were first identified due to their association with E1A. It is now clear that p107 and p130 associate with transcription factors capable of binding to the E2F DNA site (Shirodkar, et al., 1992; Devoto, et al., 1992; Cao, et al., 1992; Cobrinik, et al., 1993), thus the homology to pRb is functional as well as physical. However, the E2F proteins that associate with pRb do not appear to interact with p107 or p130 in cells. It is therefore likely that pRb, p107 and p130 are members of a family of proteins which regulate the function of a family of E2F transcription factors whose roles may or may not overlap in cell cycle control. Deregulated phosphorylation of these targets and pRb together may be more profoundly oncogenic than deletion of pRb alone, consistent with the high incidence of cyclin D1 overexpression in certain tumor subsets, such as is the case in breast cancer. We are attempting to identify the functional regions of cyclin D1 operative in transformation and correlate these with an induction of pRb phosphorylation.

Work in progress outlined in last year's progress report has demonstrated that complete elimination of the LXCXE domain in both cyclin D1 and cyclin D2 has no effect on the ability of these cyclins to activate cdk4 or cdk6 "in vitro", that is in cotransfection assays. Thus, whether pRb is supplied to cells in transfections or used as a synthetic substrate in in vitro kinase assays, complexes containing D-cyclins lacking the LXCXE domain can efficiently phosphorylate pRb.

Mutant cyclin D1 proteins lacking the LXCXE domain, the ability to activate cdk4/6, or N-terminal regions between the LXCXE domain and the cyclin box are being tested in transformation and growth arrest assays. As was discussed in the previous report, and consistent with earlier results using the point-mutant protein, cyclin D1 lacking LXCXE was found to be fully functional in transformation. In contrast, initial experiments suggested that cyclin D2 lacking LXCXE is poor at transformation, indicating possible functional differences between the two cyclins. These experiments are continuing; however, at this point it is clear that the LXCXE domain of cyclin D1 is dispensable for kinase activation, phosphorylation of pRb in vitro and transformation of primary cells.

We have employed these cyclin proteins in a second growth assay with interesting results. This assay tests the ability of the cyclin protein to disrupt growth arrest caused by the transient expression of pRb in SAOS-2 human osteosarcoma cells. These cells undergo a cell cycle arrest in G1 and a distinct morphological alteration upon reexpression of pRb (Hinds, et al., 1992). Because the morphologically altered cells ("flat cells") can be counted, this assay allows a quantitative, visual assay for the ability of a co-introduced expression construct to disrupt pRb function. Previously, it had been found that cyclins A and E could disrupt flat cell formation with concomitant induction of pRb phosphorylation. Cyclin D1 also reduced flat cell formation, but no evidence of pRb hyperphosphorylation was seen (Hinds, et al., 1992). We have begun to use this assay for D-type cyclin mutants in effort to identify the function(s) of D-cyclins that disrupt pRb function in SAOS-2 cells.

Results from SAOS-2 cotransfections show that pRb-induced flat cells can be efficiently disrupted by LXCXE point mutant cyclin D1 as previously reported (Dowdy, et al., 1993), and further, the complete disruption of the LXCXE domain has no effect on the ability of cyclin D1 to prevent growth arrest. Thus, as with transformation and phosphorylation, the LXCXE domain is dispensable in functional assays of cyclin D1. Interestingly, we find that cyclin D2 is less efficient at preventing flat cell formation in this assay. Further, the weak reduction in flat cells seen with wild-type cyclin D2 is completely lost by either point mutation or deletion of the LXCXE domain. Cyclin D1 may thus have a unique effect in this assay, independent of the LXCXE domain, and likely independent of pRb phosphorylation since we do not observe phosphorylation of pRb in these assays. Cyclin D2, on the other hand, may require the LXCXE domain for function, or may use this domain simply to compete other pRb targets from the functional domain. A further interesting point arising from these assays is that a nonfunctional cyclin D1, called KE, that cannot activate cdk4/6, nevertheless retains the ability to reduce flat cell formation caused by pRb. This data may indicate that either an LXCXE domain or catalytic competence of cyclin D1 may be sufficient to prevent flat cell formation by pRb. To test this possibility, a double mutant containing both the KE mutation and the LXCXE mutation was produced. This mutant, like its singly-mutant parents, retains the ability to prevent flat-cell formation, suggesting that a novel function of cyclin D1 may prevent this senescence-associated function of pRb. Experiments designed to extend this observation are ongoing. In addition, a KE version of cyclin D2 has recently been constructed to determine if this protein is a functional antagonist of

pRb. Finally, in addition to phenotypic alterations, pRbs ability to cause a G1 arrest has been assayed in the presence of excess D-cyclin. In this case, we have preliminary evidence that while the LXCXE domain is dispensable for reversal of growth arrest, KE is incapable of this, suggesting that kinase activation may be required to fully inactivate pRb, but that growth arrest and phenotypic alteration are separable properties of pRb that are influenced differently by cyclin D1. This type of functional distinction is potentially at the heart of the oncogenicity of cyclin D1 and is thus being pursued vigorously.

The experiments described above will result in a clear picture of the role of the LXCXE domain in antagonizing pRb function in several in vitro (cell culture) assays. In general, they suggest that this domain is dispensable for in vitro pRb kinase activity and for cyclin D1 function in proliferation. Further, results with the catalytically-inactive KE mutant of cyclin D1 suggest that a noncatalytic role of cyclin D1 might interrupt some inhibitory effects of pRb, perhaps in an indirect manner. This is an exciting possibility in light of the ability of KE to activate the estrogen receptor, and to inactivate the DMP-1 transcription factor, thought to be involved in growth arrest. However, a drawback of these experiments is that they all result from significant overexpression of D cyclins. This level of expression may override a need for the LXCXE domain that would be seen at endogenous levels of expression. We feel that the best way to examine this issue is to express cyclin D1 mutants under endogenous control mechanisms. To do this, we have produced a targeting vector that will allow integration of wild-type or mutant cyclin D1 cDNA in the mouse genomic cyclin D1 locus, disrupting this gene and allowing expression of the product of the cDNA (Figure 2). Introduction of these alleles into ES cells has begun, and identification of properly recombinant clones will allow generation of mice expressing cyclin D1 only from these engineered alleles. This, in turn, will allow us to examine the ability of mutant forms of cyclin D1 to "rescue" the phenotypes caused by disruption of the mouse cyclin D1 gene (Sicinski, et al., 1995). This work will be done in collaboration with Piotr Sicinski, who has recently joined the Harvard Medical School Pathology faculty at the Dana-Farber Cancer Institute.

Dr. Sicinski's knockout of cyclin D1 revealed that cyclin D1-deficient mice fail to develop a functional retina, have neurological problems, and perhaps most significantly, fail to develop lactating breast after pregnancy. This loss of lobuloalveolar proliferation is particularly intriguing given the major role that cyclin D1 plays in human breast cancer. By producing mice containing only mutant cyclin D1 alleles, rather than null alleles, we can assess the importance of catalytic and non-catalytic roles of cyclin D1 to these phenotypes. This is particularly important with the KE mutant, since this mutant is known to retain the ability to activate the estrogen receptor in in vitro experiments. We anticipate that this carefully controlled expression of mutant cyclin D1 will also reveal any role of the LXCXE domain in breast or retinal development and will allow the generation of cultured cells expressing the mutant protein at normal levels. These cells will allow careful comparisons of the biochemical properties of mutant cyclin D1 and better reveal the role of the LXCXE domain.

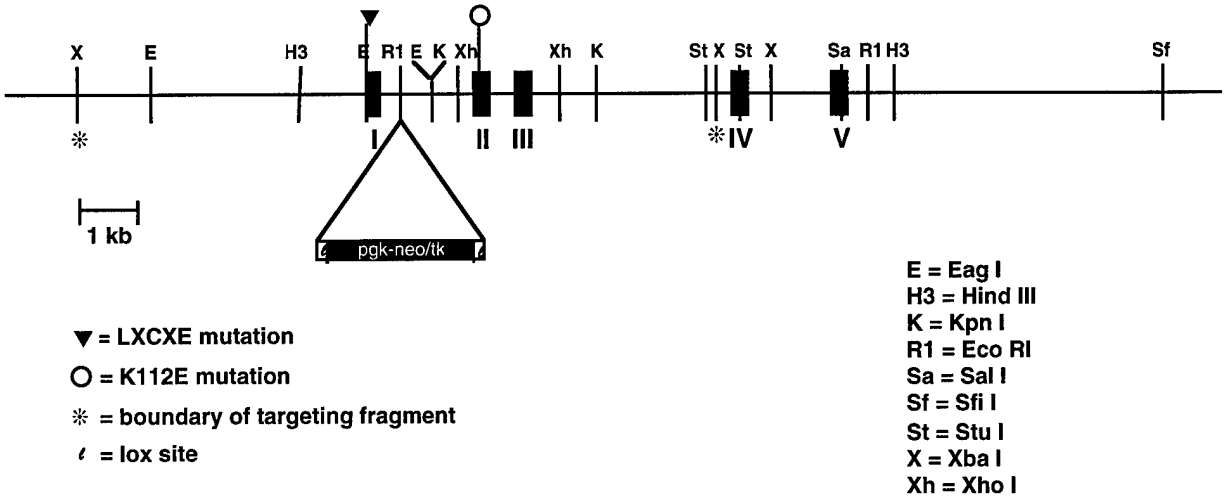

Figure 2. Structure of cyclin D1 mutant targeting fragments. The mouse cyclin D1 gene is mapped, and the sites of point mutation of the LXCXE domain or K112E are shown. A drug resistance cassette has been inserted into the Eco RI site in intron 1 and will be used to select recombinants. After determination of correct integration by Southern blot, lox sites flanking the neo/tk fusion cassette will be utilized to allow excision of the cassette after introduction of cre recombinase. The resulting locus will contain point-mutated cyclin D1 and a small insertion in intron 1.

Specific aims 3 and 4: Use of dominant-negative cyclin D1 and molecular mimics thereof.

Our previous and ongoing work has shown that the cyclin D1 mutant KE is not only inactive for kinase activation and transformation, but can act as a dominant-negative in transformation assays (Hinds, et al., 1994 and unpublished results). As part of the studies described above and in the previous report, we have found that KE can bind to cdk4 with wild-type efficiency in transfections into human tumor cells, but the resultant complex is catalytically inactive. In contrast, in E1A-transformed BRK cells, KE appears associate with kinases only weakly in comparison to the wild-type protein. Finally, work in collaboration with Dr. Steve Dowdy suggests that KE can make complexes with cdks in most cells tested; it is possible that the expression of E1A in BRK cells compromises complex formation between D cyclins and cdks and accentuates a minor difference in affinity that is usually of little consequence.

We have continued to investigate the utility of dominant-negative cyclin D1 in human tumor cells. First, we have attempted to dissect the mechanism of dominant-negativity by co-introducing KE and wild-type cyclin D1 in SAOS-2 cells along with cdk4 to demonstrate KE-induced inactivation of cdk4. Surprisingly, we found that KE could not act as a dominant negative in this assay. We surmised that excess cdk4 resulting from transfection might prevent sequestration of this kinase subunit by KE, so attempted to perform similar experiments in U2OS cells,

which express wild-type cyclin D1 and functional pRb. Here again, however, we have seen little effect of KE on U2OS cell proliferation, suggesting that cdk4 and cdk6 function is not greatly affected by KE expression. Biochemical analyses of the complexes formed in these experiments has not yet been performed, so the ability of KE to compete for cdk4 in this assay cannot be determined. Clearly, more work is required to define the conditions and properties of KE that allow it to act in a dominant fashion before the goals of these aims are achieved.

We plan to continue these studies by comparing kinase activities in co-transfected tumor cells that seem resistant to KE and in primary BRK cells, where the dominant-negative effect was first seen to determine if dominant kinase inactivation is cell-type specific. In addition, the KE mutation has been introduced into cyclin D2, as described above, where it is also inactivating. A similar effect may result from mutating the analogous residue in cyclin G1, since we observe a profound stabilization of the mutant form of this protein. In contrast, we have also placed this mutation in v-cyclin, a D-like viral cyclin and in cyclin E and have observed little effect on pRb phosphorylation in co-transfections, suggesting that the KE mutation may not inactivate all cyclin classes. Direct assays for kinase function need to be performed with all mutant cyclins to distinguish direct and indirect effects on pRb phosphorylation, but clearly further analysis of this class of cyclin mutants will be very informative and is required before further functional mimics can be identified.

Specific aim 5: Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

Considerable progress has been made in the characterization and utilization of the temperature-sensitive allele of pRb (tspRb) called XX668. Briefly, this allele encodes a pRb that can very poorly induce flat cells, G1 arrest or colony reduction at 37° C, but does so with wild-type efficiency at 32.5°C. Further, the protein cannot repress E2F-dependent promoters at 37° C but is functional for this property at 32.5°C. Most interestingly, when tspRb is inactivated by temperature upshift to the nonpermissive temperature, BrdU incorporation is seen within 24 hours, indicating that the pRb-induced block to S phase is reversible. However, these cells do not go on to proliferate as do their untransfected counterparts but rather die shortly after S phase entry. This death is apoptotic as determined by FACS analysis and the ability of bcl-2 and E1b19K to overcome the death phenotype. Thus, establishment and reversal of pRb function in SAOS-2 osteosarcoma cells does not restore the original tumorous phenotype of these cells, but results in cell death. We believe that some program or downstream function of pRb manifested after long-term (several days) expression of pRb in growth arrested cells leads to a continuous anti-proliferative signal that conflicts with the re-establishment of S phase signals upon pRb inactivation. This work has been published (Tiemann and Hinds, 1998) and a reprint is furnished in the Appendix.

We are using this tspRb in several ways. First, we are further investigating the defect in tspRb at 37°C that disallows E2F-dependent transcriptional repression. We have found that tspRb interacts with E2F equally

at both temperatures, and so an inability to bind E2F is apparently not at the heart of tspRb's inaction at 37°C. Instead, it is possible that the transcriptional repression function of the pRb pocket is specifically defective. To test this, we have constructed GAL4-DNA binding domain constructs that contain the pRb and tspRb pocket to test for E2F-dependent transcriptional repression. Using these reagents, we have found that tspRb remains temperature sensitive for transcriptional repression even when bound to GAL4 DNA binding sites, suggesting that some function of pRb other than E2F binding is disrupted at the nonpermissive temperature. Recently, histone deacetylases (HDAC) have been reported to associate with pRb and be at least in part responsible for the transcriptional repression observed when pRb is brought to a promoter. We are presently testing the ability of tspRb to associate with HDAC proteins to determine if a defect in this association could explain the temperature sensitive function of this mutant.

A second use of tspRb relies on cell lines that have been established in the SAOS-2 background. Several of these undergo growth arrest upon induction of pRb function, but only some undergo the morphological alteration characteristic of transiently transfected cells. The different phenotypes of these stable cell lines will allow us to not only examine the cell death resulting from pRb re-inactivation, but will allow us to identify genes that are turned on and off as pRb is activated and inactivated. We may thus be able to identify genes involved in proliferation that are directly controlled by pRb, but further may also identify genes involved in the phenotypic change induced by pRb. This can be achieved by comparing expression profiles in cell lines with and without morphological alteration. Studies of these cell lines will help determine if pRb is activating a differentiation program in SAOS-2 cells or is effecting terminal cell cycle exit without altering the differentiation properties of these cells. To date, our studies with two temperature sensitive pRb lines clearly shows induction of markers of senescence. Senescence-specific betagalactosidase is turned on after pRb activation, as is PAI-1, another marker of senescence. This coupled with the phenotypic change associated with pRb expression, supports the re-establishment of a senescence program in SAOS-2 cells expressing tspRb. Further characterizations will include a study of telomerase function in these cells, since inactivation of this enzyme has been associated with senescence, and indeed could be key to the irreversibility of this process.

In contrast to the clear induction of senescence markers in SAOS-2 cells, our current studies fail to support a role for pRb in induction of differentiation markers. No changes have been observed in alkaline phosphatase expression, an early marker of bone differentiation, nor do transcript or protein levels of osteocalcin or osteopontin change with pRb induction. Suitable positive controls make this negative result significant, but we plan to study the effect of pRb on the cloned osteocalcin promoter and its activator, OSF-2, using reagents now available in the lab. At present, it appears that pRb effects a terminal cell cycle program consistent with senescence in SAOS-2 cells, but does not cause differentiation. Using these cells as a source for messenger RNA, we can now attempt to identify genes regulated by pRb in its role as a senescence-inducer. We anticipate initially using standard subtractive hybridization techniques to achieve

these goals, but will consider the SAGE technique if cost allows. Identification of these potential pRb-regulated genes will be very important in understanding the exact role of pRb in cancer as well as in elucidating the molecular basis of senescence.

Finally, we wish to introduce tspRb into other cell types in attempt to identify cell type specific changes caused by pRb. Indeed, the apparent senescence-specific role of pRb in SAOS-2 cells does not preclude a differentiation function in other cell types. We plan to introduce tspRb into MDA-MB-468 breast tumor cells which lack functional pRb. A functional analysis of such transfectants could be quite useful in elucidating a role for pRb in terminal differentiation of breast epithelial cells.

CONCLUSIONS

Our ongoing work characterizing the method of action of cyclin D1 as an oncogene will be useful in identifying the functions of cyclin D1 that may serve as targets for anti-tumor approaches. Preliminarily, it seems that cyclin D1 differs from cyclin D2 in its biochemical effects on pRb in overexpression systems. These differences may explain the common observation of cyclin D1 as a human oncogene, in contrast to the rare incidence of cyclin D2 overexpression. Such differences are what we hope to exploit to reverse the aberrant proliferation resulting from cyclin D1 overexpression. The expression of cyclin D1 mutants in animal model systems seems necessary to achieve a clear understanding of the full role of cyclin D1 in proliferation. The ability to study this in breast development and other phenotypic consequences is exciting and made possible by a collaboration with Piotr Sicinski.

TspRb shows great promise as a reagent to unravel the specific effects of pRb on terminal cell cycle exit. Our present results with this reagent suggests that tspRb can cause a readily reversible block to S phase entry, but also causes more durable changes in the cell that result in death once pRb is inactivated. Such properties, if also seen in other cell types, could be quite useful in identifying gene targets and properties of pRb that influence terminal cell cycle exit and differentiation.

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Induction of DNA synthesis and apoptosis by regulated inactivation of a temperature-sensitive retinoblastoma protein

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The retinoblastoma protein, pRb, controls entry into the S phase of the cell cycle and acts as a tumor suppressor in many tissues. Re-introduction of pRb into tumor cells lacking this protein results in growth arrest, due in part to transcriptional repression of genes required for S phase. Several studies suggest that pRb may also be involved in terminal cell cycle exit as a result of the instigation of senescence or differentiation programs. To understand better these multiple growth-inhibitory properties of pRb, a temperature-sensitive mutant of pRb has been produced. This tspRb induces G_1 arrest and morphological changes efficiently at the permissive temperature of 32.5°C, but is weakly functional at 37°C. Consistent with this, tspRb is compromised in nuclear association and E2F regulation at the non-permissive temperature, but regains these properties at 32.5°C. Serial activation and inactivation of tspRb in SAOS-2 cells does not allow proliferation, but rather leads to apoptotic cell death. Transient activation of pRb may kill tumor cells by establishing a conflict between persistent proliferation-inhibitory signals and renewed deregulation of pRb targets such as E2F, and may thus be a more potent means of eliminating these cells than through simple re-introduction of the tumor suppressor gene.
Keywords: apoptosis/E2F/growth suppression/pRb/temperature-sensitive mutant

Introduction

The retinoblastoma susceptibility protein, pRb, functions in a cell cycle control pathway that regulates passage of cells through G_1 and into S phase (Weinberg, 1995). pRb exerts its function in part by controlling a family of heterodimeric transcription factors, collectively referred to as E2F, that can transactivate genes important for the G_1 to S phase transition. Hypophosphorylated pRb binds to E2F, resulting in a transcriptional repressor complex that constrains expression of E2F target genes. Phosphorylation of pRb in mid to late G_1 releases E2F from the complex, which now can be transcriptionally active (Weinberg, 1995; Sherr, 1996). Targeted deletion of the E2F-1 gene suggests that E2F-1 is necessary for the proliferation of some cells, but actually acts as a tumor suppressor in other cell types. This dual role of E2F-1 may result from an ability of E2F-1–pRb complexes to repress genes involved in apoptosis as well as genes whose products are needed for DNA synthesis (Qin *et al.*, 1994; Field *et al.*, 1996; Yamasaki *et al.*, 1996; Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

Phosphorylation of pRb and concomitant relief of E2F repression is catalyzed by cyclin D-dependent kinases (cdk4 and 6) in response to extracellular signals. The activity of these kinases is in turn inhibited by proteins of the INK4 family (Sherr, 1996). Disruption of this so-called 'pRb pathway' is a frequent event during the pathogenesis of a variety of human tumors (Tiemann *et al.*, 1997), highlighting the importance of the integrity of this pathway in the prevention of tumor formation, as well as pRb's central role in it.

The function of pRb is lost in all retinoblastomas, where it can lead to hereditary cancer, but is also involved in a variety of other tumors, including small cell lung carcinomas, breast carcinomas, osteosarcomas, bladder carcinomas, prostate carcinomas and cervical carcinomas (Dyson *et al.*, 1989; Horowitz *et al.*, 1990; zur Hausen, 1991). Several different types of inactivating mutations in the *RB1* gene have been described, almost all of which specifically alter or eliminate the 'pocket' domain of pRb. The pocket domain was first functionally characterized as the minimal region of pRb that is necessary for binding to the viral oncoproteins E1A, E7 and large T antigen. Thus, both oncogenic mutations and viral oncoprotein binding target the same subdomain of pRb, strongly suggesting a critical role for this domain in tumor suppression (Tiemann *et al.*, 1997). Indeed, the pocket domain is sufficient for the repression of gene expression, but an extra C-terminal stretch of the pRb protein is necessary for E2F binding and growth suppression (Qin *et al.*, 1992; Hiebert, 1993). pRb shares the pocket domain with two other related proteins, p107 and p130, which also bind and regulate E2F activity, but which have not yet been shown to be mutated in cancer (Ewen *et al.*, 1992; Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993; Mayol *et al.*, 1993).

Although the pRb/E2F paradigm leads to a simple model for growth regulation through the inactivation of E2F-responsive genes, the identity of genes actually regulated by pRb is far from fully elucidated. The presence of p107 and p130 together with pRb in all cells complicates any simple model of pocket protein–E2F function, since these molecules may regulate subsets of E2F site-containing genes differentially (Hurford *et al.*, 1997). Further, pRb interacts with many proteins besides E2F, several of which can themselves directly influence gene expression (Taya, 1997). Among these are differentiation-specific transcription factors that are activated by pRb, rather than repressed, emphasizing a potential role for pRb in the process of terminal differentiation. Additionally, there is evidence that pRb may inhibit apoptosis (Haas-Kogan *et al.*, 1995; Berry *et al.*, 1996; Fan *et al.*, 1996; Macleod

et al., 1996; Shan *et al.*, 1996; Hsieh *et al.*, 1997) and somehow regulate replicative senescence, a phenomenon antithetical to tumor cell formation (Smith and Pereira-Smith, 1996).

In vivo evidence for a role for pRb in terminal cell cycle exit comes from mouse models in which both *RB1* alleles have been replaced with defective alleles by means of homologous recombination techniques. Mice homozygous for defective *RB1* die *in utero* at about the fourteenth day of gestation from abnormalities in the blood and the liver accompanied by extensive cell death in the central nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Although pRb is also absent in all other tissues of the mouse embryo, most tissues develop grossly normally. Nevertheless, loss of pRb may lead to more subtle alterations in differentiated cell properties that have unknown consequences because of the inability to study them in dead embryos. For example, although muscle development appears normal in *RB1* nullizygotes, myotubes produced from such embryos fail to enter a truly 'terminal' state, since their nuclei can be induced to enter S phase, unlike those from genetically normal mice (Schneider *et al.*, 1994; Novitch *et al.*, 1996).

Multiple effects of pRb are also evident in cell culture model systems. For example, cells of the human osteosarcoma cell line SAOS-2 undergo a G_1 growth arrest upon pRb expression that is accompanied by a distinctive morphological change (Huang *et al.*, 1988; Templeton *et al.*, 1991; Hinds *et al.*, 1992). This phenotype is characterized by spreading and a senescence-like morphology (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Qin *et al.*, 1992). This morphological alteration is not seen with dominant-negative E2F proteins or p107 overexpression, despite the probable mechanistic similarities in their ability to block progression into S phase (Zhu *et al.*, 1993; Qin *et al.*, 1995). Therefore, pRb may influence the expression of genes controlling cell morphology in a manner distinct from simple repression of E2F-dependent promoters.

In an attempt to explore the biochemical basis of pRb's functions and to produce a system to identify potential gene targets of pRb, we have generated a temperature-sensitive (ts) pRb. Characterization of this mutant supports a multifunctional role for pRb in suppression of cell proliferation, identifying both reversible and irreversible elements of growth arrest engendered by pRb. We suggest that pRb expression in this system not only prevents E2F function, but produces (possibly E2F-independent) cellular changes akin to differentiation or senescence that cannot be overcome by simple loss of pRb function, leading instead to apoptosis when the cells are once again rendered pRb-null through temperature shift. Transient restoration and removal of the differentiation and proliferation controls exerted by some tumor suppressor proteins may thus be a more potent method to eliminate tumor cells than that afforded by constitutive expression.

Results

Identification of a pRb protein temperature-sensitive for the induction of growth arrest

Regulated ectopic expression of genes involved in proliferation control is useful for studying their immediate effects upon synthesis and loss. For example, both inducible and temperature-sensitive p53 genes have been useful in identifying functional and transcriptional targets of this important tumor suppressor (Michalovitz *et al.*, 1990; Yonish-Rouach *et al.*, 1991; Barak *et al.*, 1993; El-Deiry *et al.*, 1993; Okamoto and Beach, 1994; Wu and Levine, 1994; Chen *et al.*, 1996). Because pRb's long half-life makes it an unattractive candidate for inducible systems, we attempted to identify a temperature-sensitive version of the protein that could be regulated more rapidly. Therefore, a series of *in vitro* mutagenized pRb cDNAs was produced (Mittnacht *et al.*, 1991; Hinds *et al.*, 1992) and tested for growth suppression in SAOS-2 cells at 37 and 32.5°C. As shown in Figure 1, the four linker-insertion mutants and the tumor-derived negative mutant Δ22 (Horowitz *et al.*, 1990) were found to be inactive in growth suppression at 37°C, but one mutant was found to induce growth suppression at 32.5 but not at 37°C. This mutant, XX668, carries a 12 bp *Xho*I linker inserted in-frame into an *Xmn*I site in between codons 668 and 669. This results in the insertion of four new amino acids, RSSG, changing the sequence of this mutant at codon 668 to -er[RSSG]ll- (see Figure 1).

Protein	Mutation	Growth suppression at 37 °C	Growth suppression at 32.5 °C
HX108	RV**PLER**DL (108)	-	-
AcX414	S**IPRG**ILK (414)	-	-
XX668	ER**RSSG**LL (668)	-	+
AX763	Q**PLE**RL (763)	-	-
wtpRb	-	+	+
Δ22	deletion of exon 22	-	-

Fig. 1. Structure of *in vitro* mutagenized pRb proteins and their ability for growth suppression at 37 and 32.5°C. *In vitro* mutants were constructed by oligonucleotide linker insertion into restriction sites at certain codons of the human *RB1* cDNA. All mutants have been tested to express mutated forms of the pRb protein. The schematic diagram shows the human pRb protein and the positions where the *Xho*I linker was inserted in-frame to generate the mutants HX108, AcX414, XX668 and AX763. The solid black boxes A and B represent the two regions of the retinoblastoma protein essential for binding to E1A and SV40 large T antigen (Hu *et al.*, 1990), together known as the pocket domain of pRb. The shaded gray box represents the nuclear localization signal (NLS) of pRb (amino acids 860–876). To determine the ability of the generated *in vitro* mutants to suppress growth at 37 or 32.5°C, SAOS-2 cells were transfected with pBabepuro and HX108, AcX414, XX668, AX763, wtpRb or Δ22, and transfected cells were selected at 37 or 32.5°C. Suppression of SAOS-2 cell growth was assessed by the appearance of large, apparently non-dividing cells 7–10 days after transfection. (+) Large number of such cells. (–) No or very few enlarged cells were observed.

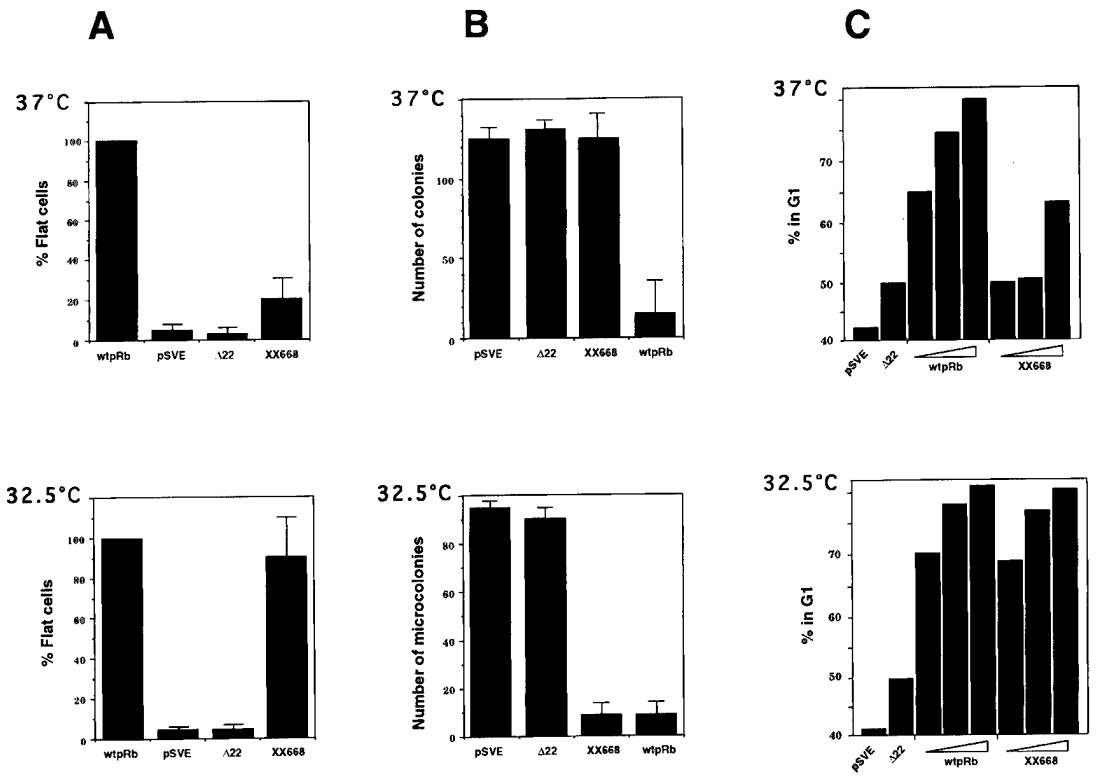

Fig. 2. Temperature-dependent growth suppression by the pRb mutant XX668. For flat cell assays (**A**) and colony formation assays (**B**), cells were transiently transfected with 1.5 μg of pBabepuro and 20 μg of the indicated pRb expression plasmid. Following transfection, cells were plated at 5×10^5 per 10 cm dish, subjected to drug selection at either 37 or 32.5°C and stained 7–10 days (A) or 14 days (B) later. Flat cells (A) or colonies (B) were counted as described in Materials and methods. The graphs (A and B) represent the mean of at least three independent experiments. For the flat cell assays (A), the percentage of flat cells relative to wtpRb (100%) is given in the graph. For cell cycle analysis (**C**), cells were co-transfected with 2 μg of pCMVCD20 and 20 μg of pSVE or Δ22, or 5, 10 and 20 μg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20 μg) by adding pSVE plasmid DNA, where needed. Cells were incubated at 37 or 32.5°C and harvested 48 h post-transfection, stained for CD20 and DNA content, and analzyed by flow cytometry. The percentage of CD20-positive cells in G_1 was plotted for each sample. The data presented are representative of multiple experiments.

One of the hallmarks of pRb-induced growth arrest in SAOS-2 cells is a distinctive morphological change which has been referred to as the flat cell phenotype (Hinds *et al.*, 1992). This phenotype is characterized by spreading and a senescence-like morphology (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Qin *et al.*, 1992). Quantitation of flat cell induction, shown in Figure 2A, revealed that XX668 at 37°C produces on average 20% of the number of flat cells produced by wild-type pRb (wtpRb), but is essentially equivalent to wtpRb in flat cell production at 32.5°C. As a further measure of the ability of XX668 to suppress proliferation, colony formation assays were performed. Figure 2B demonstrates that XX668 suppressed outgrowth of SAOS-2 cell colonies with wild-type efficiency at 32.5°C, but was unable to do so at 37°C. To demonstrate conclusively that XX668 behaves as a temperature-sensitive allele not only in the production of flat cells but also in the induction of G_1 arrest in SAOS-2 cells, we used a modified flow cytometry technique that was reported previously for pRb and p107 (Zhu *et al.*, 1993). XX668 or control vectors were co-transfected into SAOS-2 cells with a plasmid expressing the cell surface marker CD20. Two days after transfection, cells that contained transfected DNA were identified by staining with an anti-CD20 monoclonal antibody, and the DNA content of the transfected cells was determined by propidium iodide staining. As shown in Figure 2C, transfection of XX668 was unable to alter the G_1 fraction of SAOS-2 cells efficiently at 37°C, whereas even low amounts of the wtpRb cDNA led to a significant increase in the G_1 population. A slight increase in the G_1 population was seen when XX668 was transfected in a very high concentration, consistent with its partial ability to induce flat cell formation. In contrast, at 32.5°C, XX668 and wtpRb both blocked SAOS-2 cells very efficiently in the G_1 phase of the cell cycle. Thus XX668 is defective in flat cell formation, colony reduction and induction of G_1 arrest when expressed at 37°C, but is indistinguishable from wtpRb at 32.5°C. In contrast, the tumor-derived mutant Δ22 was found to be non-functional in these assays at either temperature (see Figure 2A–C), consistent with previous reports classifying Δ22 as a 'loss-of-function' mutant (Horowitz *et al.*, 1990; Templeton *et al.*, 1991).

To exclude the possibility that the observed differences of XX668 in growth suppression and flat cell formation at 32.5 and 37°C may be attributable to differences in transfection efficiencies or expression levels rather than to a functional temperature-sensitivity, we performed indirect immunofluorescence and immunoblotting. In a typical experiment, regardless of the temperature or of whether XX668 or wtpRb cDNA was used, ~30% of the transfected SAOS-2 cells became pRb positive (data not shown). In

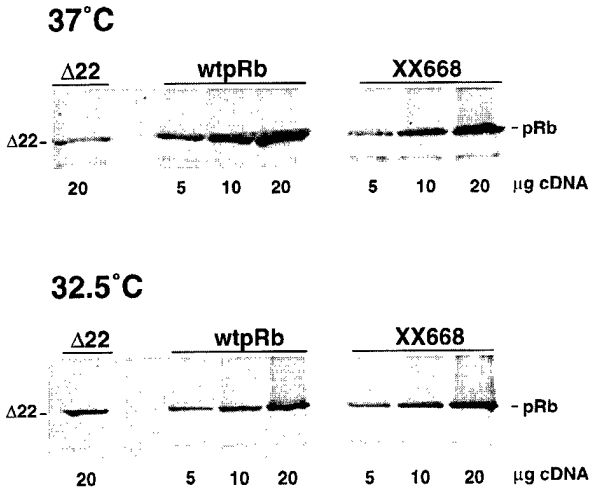

Fig. 3. Expression of wild-type and mutant pRb cDNAs in SAOS-2 cells. SAOS-2 cells, transfected as described in Figure 2B with the indicated amounts of Δ22, wtpRb or XX668 and incubated for 48 h post-transfection at 37 or 32.5°C, were lysed and aliquots were immunoblotted with an anti-pRb monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein are indicated.

addition, protein levels expressed from increasing amounts of XX668 or wtpRb cDNA were essentially equal at both temperatures (Figure 3).

These data suggest that XX668 encodes a mutant pRb protein that is essentially wild-type for suppression of proliferation and flat cell formation at 32.5°C, but is only very weakly functional at 37°C, despite its persistence in the cell. XX668 therefore can be considered as a temperature-sensitive allele of *RB1*.

Biochemical properties of tspRb

We explored the biochemical properties of tspRb in an effort to understand the defect that inactivates the protein at the non-permissive temperature. Using an *in vitro* binding assay, we first determined if tspRb's ability to associate with the pocket-binding viral oncoprotein E1A could correlate with its temperature-dependent growth-suppressing function. Extracts from SAOS-2 cells transfected with either XX668, wtpRb or Δ22, and cultured at 37 or 32.5°C, were mixed with E1A that was made as a GST fusion protein in *Escherichia coli* and incubated at 4°C. The fraction of pRb that was bound by the GST–E1A protein was visualized by immunoblot with a pRb-specific monoclonal antibody (Figure 4). Equal amounts of input pRb protein were assured by performing direct immunoblots with aliquots of the cell extracts used in the *in vitro* binding experiments (Figure 4, Input). Regardless of the temperature at which transfected cells had been cultured, only ~10% of tspRb was associated with the E1A oncoprotein when compared with wtpRb. Similar results were observed when *in vitro* binding was performed at 25 or 37°C (data not shown). Thus, the four amino acid insertion in the B-pocket of tspRb severely compromises the ability of the mutant protein to associate with E1A at both the permissive and the non-permissive temperatures. Further, based on the assay employed, there is no correlation between tspRb's ability to suppress cell growth and its ability to bind to the E1A oncoprotein.

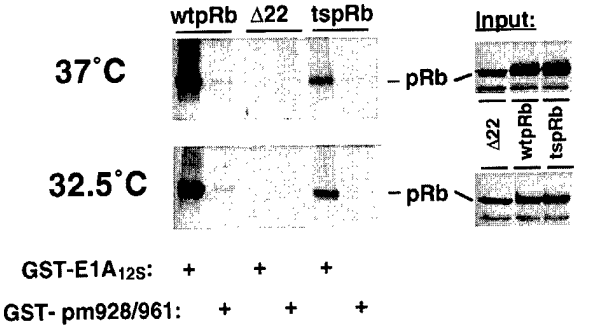

Fig. 4. *In vitro* binding of pRb proteins to E1A. SAOS-2 cells were transfected with 20 μg of Δ22, wtpRb or tspRb (XX668) and maintained for 48 h post-transfection at 37 or 32.5°C. Aliquots of the different cell lysates were incubated with glutathione–Sepharose loaded with GST–$E1A_{12S}$ or GST–pm928/961. Bound proteins were separated by SDS–PAGE and immunoblotted for pRb. To control the input of the pRb proteins, aliquots of the same lysates were immunoblotted directly for pRb (Input).

In contrast to E1A binding, nuclear association of pRb is well correlated to its functional state (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Templeton *et al.*, 1991). Functionally active pRb stably associates with nuclear structures, whereas pRb that has been inactivated by hyperphosphorylation or mutation fails to do so (Mittnacht and Weinberg, 1991). This biochemical property of pRb can be measured by treatment of the transfected cells with a detergent-containing buffer of low salt concentration. Under these conditions, functional pRb is retained in the nucleus, but inactive pRb is extracted (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1994). To examine this property of tspRb, SAOS-2 cells were transfected with XX668, wtpRb or control vectors, cultured for 2 days at 32.5 or 37°C and finally lysed in a hypotonic buffer containing 0.1% Triton X-100. Lysates then were fractionated by low-speed centrifugation into a low-salt-soluble supernatant and an insoluble nuclear pellet fraction. Distribution of the pRb proteins to the fractions was determined by immunoblotting and is shown in Figure 5. As expected, wtpRb was found predominantly in the nuclear pellet fraction at both temperatures, whereas the majority of the tumor-derived mutant Δ22 was extracted into the soluble supernatant (low-salt extract). In contrast, tspRb was stably associated with the nucleus and therefore was detectable mainly in the nuclear pellet fraction at 32.5 but not at 37°C, where it was extracted efficiently into the low-salt fraction. Thus, tspRb encoded by XX668 is temperature-sensitive for nuclear tethering and, consistent with previous studies of tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Kratzke *et al.*, 1994), this activity correlates well with its growth-suppressing function.

Viral oncoprotein binding to pRb is thought to mimic and to compete with the association of cellular proteins that can bind to the pocket domain of pRb. The best understood cellular targets of pRb are the members of the E2F family of transcription factors. Since physical association between E2F and pRb is postulated to result in transcriptional repression and growth arrest, we wished to determine if the defects in E1A binding, growth suppression and nuclear tethering displayed by tspRb were associated with an inability to suppress transcription and/

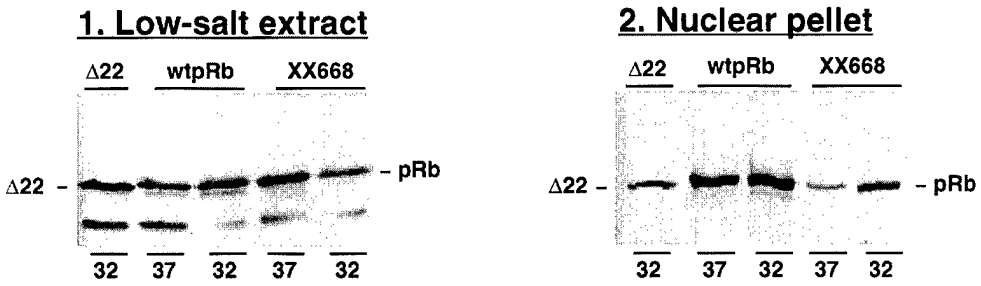

Fig. 5. Nuclear association of pRb. SAOS-2 cells were transfected with 20 μg of the indicated pRb expression construct and incubated at either 37 or 32.5°C. At 48 h after the removal of DNA precipitates, transfected cells were subjected to subcellular fractionation. Aliquots of the low-salt extract fraction and the nuclear pellet fraction were analyzed for pRb by immunoblotting with an anti-pRb-specific monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein are indicated.

or bind to E2F. To determine the functional consequence of tspRb expression on E2F-dependent transcription, we employed a reporter consisting of the luciferase gene driven by the E2F-1 promoter. The E2F-1 promoter fragment used in this experiment has been shown to be subject to repression by pRb (Sellers *et al.*, 1995). This reporter construct was co-transfected with tspRb cDNA, wtpRb or control plasmids into SAOS-2 cells, and the cells were incubated at either 37 or 32.5°C. As shown in Figure 6A, increasing amounts of tspRb were much less effective in repressing the E2F-1 promoter at 37°C, but gained this ability with wild-type efficiency at 32.5°C. This result clearly demonstrates a temperature-dependent activity of tspRb in suppression of E2F-dependent transcription.

To determine if the inability of tspRb to repress transcription through E2F is due to an inability of this mutant protein to interact with E2F, we obtained an antibody against E2F-4 (kind gift of J.Lees, MIT, Cambridge, MA) and used this to immunoprecipitate E2F-4 from SAOS-2 cells transfected with the various pRb expression constructs. Because E2F-4 is the most abundant family member in SAOS-2 cells, we anticipated that pRb–E2F-4 complexes would be detectable without further overexpression of E2F-4 (J.Lees, personal communication). Figure 6B presents the results of an anti-pRb immunoblot of the anti-E2F-4 immunoprecipitate. Whereas Δ22 is incapable of associating with endogenous E2F-4, a clear signal is obtained when wtpRb is produced in the transfectants. Transfection with XX668 yields a tspRb protein that is clearly capable of interacting with E2F-4 at 37°C, but at a reduced level. This binding does not increase when the protein is produced at 32.5°C. These data suggest that tspRb binds E2Fs more weakly than wtpRb, and this binding is not temperature-sensitive. Thus it is possible that sufficient pRb–E2F complex is present to repress E2F-dependent transcription, but repression only occurs at 32.5°C, when tspRb gains transcriptional repressor ability through an as yet undetermined mechanism.

Inactivation of tspRb leads to DNA synthesis and apoptotic cell death

The data obtained so far clearly demonstrate that tspRb in SAOS-2 cells can induce cell cycle arrest, a senescence-like morphology and E2F repression in a temperature-dependent manner. We next wished to determine if tspRb produced at the permissive temperature could be inactivated by a shift to the non-permissive temperature, and ultimately wished to study the reversibility of the pRb phenotype. To determine if active tspRb can be inactivated after transient transfection of SAOS-2 cells, tspRb-induced, growth-arrested flat cells produced by a 1 week incubation at the permissive temperature were shifted back to the non-permissive temperature and scored for re-entry into S phase by measuring the ability to incorporate bromodeoxyuridine (BrdU) (Figure 7). Flat cells produced by wtpRb were found to be incapable of incorporating BrdU at either temperature, consistent with their arrest in G_1 (or G_0). Similarly, tspRb-produced flat cells at 32.5°C could not incorporate this nucleotide. Strikingly, when the latter were shifted to 37°C, ~30% of the cells were found to incorporate detectable amounts of BrdU as early as 24 h after the shift. In contrast, growth-arrested cells produced by wtpRb expression at 32.5°C did not regain an ability to incorporate BrdU upon temperature upshift. Parental SAOS-2 cells permanently cultivated at 32.5 and 37°C and XX668-transfected SAOS-2 cells permanently cultivated at the non-permissive temperature were used as a positive control for BrdU incorporation (Figure 7). Thus, the block to S phase entry caused by tspRb appears to be rapidly reversible by temperature shift.

The apparent reversibiliy of the S phase block induced by tspRb allowed us to ask if the transiently growth-arrested SAOS-2 cells could re-enter the cell cycle permanently and/or reverse the senescence-like morphology. Incorporation of BrdU into the tspRb-transfected, temperature-shifted SAOS-2 cells occurred while the cells still possessed the flat cell morphology. To determine if inactivation of tspRb would allow the cells to regain their normal shape and proliferation capacity, flat cells were produced by transient transfection of SAOS-2 cells with XX668 and wtpRb followed by drug selection for 7–10 days at 32.5°C. Flat cells were then removed from the drug and either further incubated at 32.5°C or shifted to 37°C. Photographs of cells within a fixed area of 0.25 cm^2 were taken and the cells were counted on seven consecutive days (see Table I). While the number of wild-type transfected cells at both temperatures as well as tspRb-expressing cells at 32.5°C remained unchanged, tspRb-expressing flat cells at 37°C quickly disappeared from the plate, with loss of ~50% of the cells occurring within 24 h and loss of all cells occurring within 7 days after temperature shift. Periodic visual inspection of the remaining flat cells suggested that nuclear division commonly precedes the death of these cells, perhaps consistent with their ability to re-enter S phase prior to death

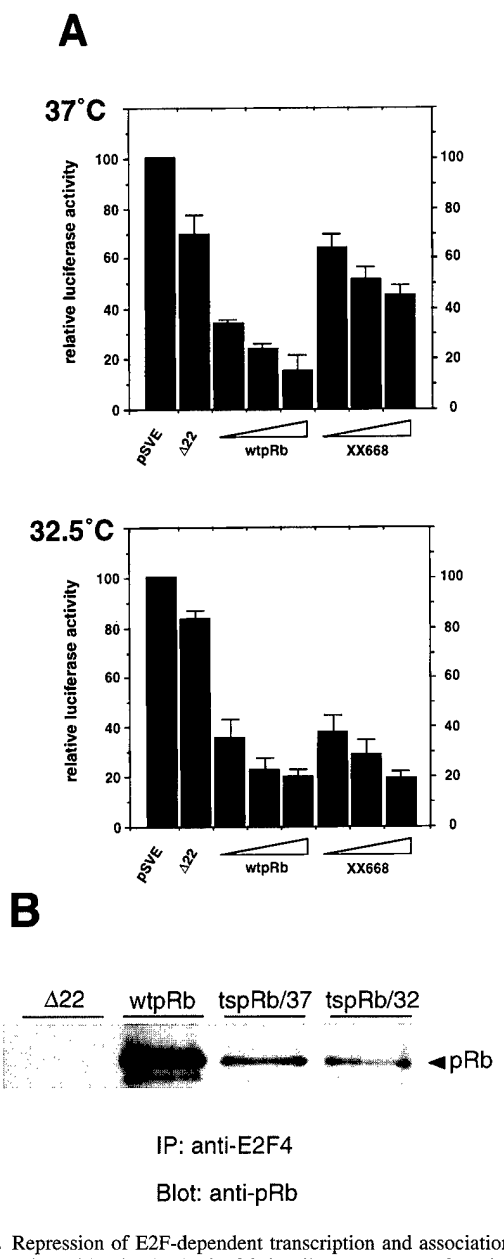

Fig. 6. Repression of E2F-dependent transcription and association of pRb proteins with E2F-4. (**A**) SAOS-2 cells were co-transfected with 5 μg of pGL2-AN, 2 μg of pCMV-βgal and 20 μg of pSVE or Δ22, or 5, 10 and 20 μg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20 μg) by adding pSVE plasmid DNA, where needed. Following transfection, cells were incubated for 48 h at 37 or 32.5°C, cell extracts were prepared and β-galactosidase and luciferase activities were assayed. Luciferase values were normalized for β-galactosidase activity and plotted relative to the activity observed for the reporter in the presence of pSVE. Data shown are representative of three experiments. (**B**) SAOS-2 cells were transfected with the indicated pRb-expressing constructs, incubated at either 37 or 32.5°C for 48 h and then subjected to lysis. Soluble proteins were immunoprecipitated with an anti-E2F-4 monoclonal antibody and the immunoprecipitates were subjected to immunoblot with anti-pRb antibodies.

(data not shown). Thus, flat cells produced by transient transfection with tspRb at the permissive temperature appear to display a reversible block to S phase entry, but are terminally blocked from proliferation, since they die after shift to the non-permissive temperature.

Because pRb loss has been reported to induce apoptosis (Lee *et al.*, 1994; Maandag *et al.*, 1994; Morgenbesser *et al.*, 1994; Macleod *et al.*, 1996; Zacksenhaus *et al.*, 1996), we wished to determine if the cell death we observed was apoptotic in nature. Therefore, we first attempted to block cell death by introducing Bcl-2 or adenovirus E1B 19K protein along with pRb. Interestingly, co-transfection of these anti-apoptotic genes along with tspRb prevented cell death upon temperature upshift (see Table I), suggesting that the observed cell death is apoptotic.

To quantitate the percentage of apoptotic cells within the total flat cell population, we determined the DNA content of the affected cells by flow cytometry analysis following propidium iodide staining (Figure 8). The percentage of apoptotic cells in the wtpRb-induced flat cell population was very low, usually <10%, regardless of the incubation temperature. Similary, flat cells produced with tspRb at 32.5°C did not contain very many apoptotic cells. In contrast, when the latter were shifted to 37°C, a significant number of cells with a sub-G_1 DNA content was observed 24 h after the shift, representing some 40% of the total cell population. At 48 h post-shift, the percentage of apoptotic cells was a little lower, but still significant at 30–40% of the total cell population. Co-introduction of either Bcl-2 or E1B 19K led to a 3- to 5-fold decrease in apoptotic cells. Furthermore, as a consequence of the additional expression of either apoptosis inhibitor, small, proliferating SAOS-2 cells, positive for the expression of tspRb, were generated (Figure 9).

Thus, cellular changes caused by prolonged pRb expression appear to preclude proliferation (but not cell cycle progression) upon removal of pRb as a result of programed cell death. Inhibition of this death by known protein inhibitors of apoptosis allows the outgrowth of colonies of pRb-positive, morphologically 'normal' SAOS-2 cells, suggesting that the flat cell phenotype can be reversed once the apoptotic signals are overcome.

Discussion

We have produced a genetically engineered pRb mutant, XX668, carrying an insertion of four amino acids at codon 668 in the B-pocket, that can exert very diverse effects on transiently transfected SAOS-2 cells, depending on the temperature at which the culture is maintained. At 37°C, despite its persistence in the cell, XX668, unlike wtpRb, is defective in flat cell formation, colony reduction and induction of G_1 arrest. In this respect, it behaves like tumor mutants described here and in earlier work (Templeton *et al.*, 1991; Hinds *et al.*, 1992). However, unlike these tumor mutants, XX668 was found to be indistinguishable from wtpRb for suppression of proliferation when the cells were kept at 32.5°C. Furthermore, at this temperature, it can induce formation of morphologically altered 'flat' cells very efficiently. Thus, XX668 encodes a mutant pRb protein that is essentially wild-type at the low temperature, but is only very weakly functional at 37°C. These findings strongly suggest that XX668 is a temperature-sensitive mutant of pRb.

Not surprisingly, the insertion in the B-pocket of tspRb severely compromised its ability to interact with E1A. tspRb interacts only weakly with E1A at 37°C, and this binding cannot be improved upon changing the

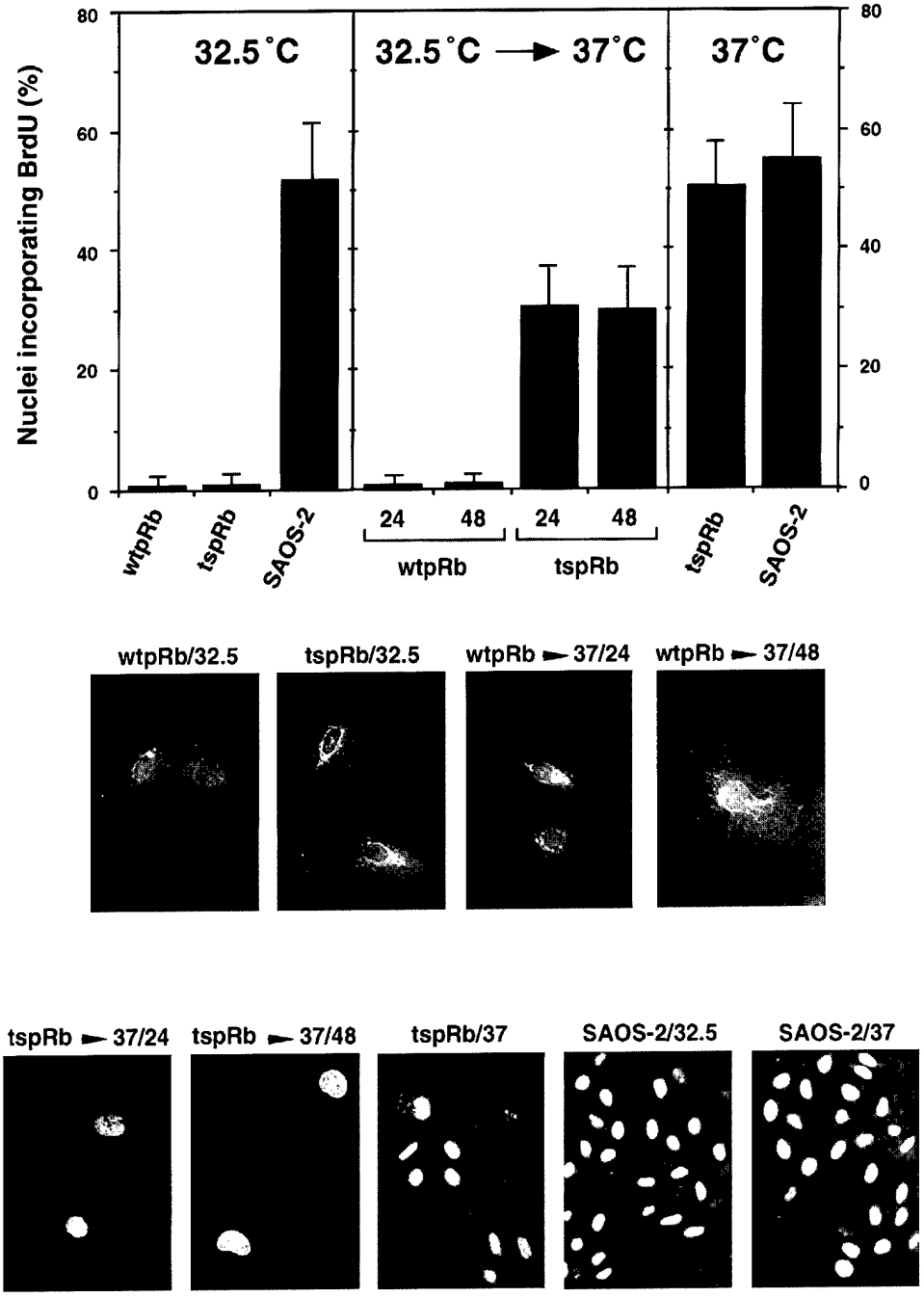

Fig. 7. Re-entry of tspRb-induced growth-arrested flat cells into S phase upon temperature shift to the non-permissive temperature. SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668). Following transfection, cells were subjected to drug selection at 32.5°C. In addition, tspRb-transfected cells were selected at 37°C. After 7–10 days, when only flat cells appeared on the plates, cells were removed from the drug and either incubated further at their former temperature or shifted from 32.5 to 37°C for 24 or 48 h. Shifted and indicated control cells were scored for re-entry into S phase by measuring their ability to incorporate BrdU. The percentage of BrdU-incorporating cells for each sample is shown in the graph. The results shown represent three independent experiments and the error bars indicate the standard deviations. Representative pictures of all samples stained for BrdU were taken at the same magnification.

temperature at which the protein is produced. Therefore, binding to E1A seems not to correlate with pRb's ability to suppress proliferation. This is consistent with previous studies in which pRb mutants with mutations within the B-pocket were reported that retained the ability to suppress cell proliferation but failed to associate with E1A (Mittnacht *et al.*, 1991; Kratzke *et al.*, 1994). A caveat to these E1A-binding experiments is that although tspRb proteins were produced in cells incubated at either the permissive or non-permissive temperature, *in vitro* binding was performed at 4°C, raising the possibility that the temperature-sensitive nature of the interaction might be altered by exposure of tspRb to the low temperature. Several observations argue against this possibility. First, tspRb produced in cells incubated at either temperature displayed equivalent *in vitro* binding to E1A (and equivalent E2F-4 co-immunoprecipitation) rather than showing an increase at the functionally permissive temperature.

Table I. Inactivation of pRb after flat cell induction results in cell death

Flat cell phenotype induced at 32.5°C with[a]	No. of cells (%) after further incubation at 32.5°C[b]		No. of cells (%) after temperature shift to 37°C[b]			
	Day 0	Day 7	Day 0	Day 1	Day 2	Day 7
Wild-type pRb	100	100	100	100	100	100
tspRb	100	100	100	55	45	0–1
tspRb + Bcl-2	100	100	100	100	100	150
tspRb + E1B 19K	100	100	100	100	100	140

[a]SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668) and 5 μg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7–10 days, when only flat cells appeared on the plate, cells were removed from the drug and either incubated further at 32.5°C or shifted from 32.5 to 37°C for up to 7 days.
[b]Average cell numbers based on four experiments were calculated (see Materials and methods) and are presented relative to the cell numbers on day 0 of either temperature which were set as 100%.

This argues that the starting pools of conformationally wtpRb were equivalent, even if an effect of 4°C incubation might lead to partial restoration of pRb binding function. Second, incubation of GST–E1A with pRb-containing lysates at 25 or 37°C failed to increase the difference between wild-type and tspRb (data not shown), demonstrating that the temperature to which tspRb is subjected *in vitro* does not necessarily influence its E1A-binding ability. Finally, the XX668 mutant is also severely compromised in its ability to associate with the nucleus at 37°C, a loss of function common to inactive, tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Templeton *et al.*, 1991; Kratzke *et al.*, 1994) but, in contrast to E1A binding, tspRb produced at 32.5°C regains the ability to associate with the nucleus, suggesting a temperature-dependent structural alteration of the pocket region required for nuclear tethering. Because this property of tspRb is also assayed at 4°C, it is clear that some physical differences between tspRb produced at permissive and non-permissive temperatures persist *in vitro*.

It is believed that transcriptional repression of genes containing E2F sites mediated by complexes of pRb and E2F contributes significantly to pRb's role as a tumor suppressor. Consistent with this, we find that tspRb is much less effective than wtpRb in repressing the E2F-1 promoter at the non-permissive temperature, but gains this ability with wild-type efficiency at 32.5°C. This result clearly suggests a temperature-dependent activity of tspRb in suppression of E2F-dependent transcription, correlating with its nuclear tethering and proliferation-suppressing activity. Interestingly, our data suggest that the inability of tspRb to suppress transcription at the non-permissive temperature is not due simply to a defect in association with E2F, since we have observed equivalent levels of pRb–E2F complexes at both the permissive and non-permissive temperatures. The XX668 mutation may thus be somewhat similar to a recently identified, low penetrance allele of *RB1*, called 661W, that is defective for E1A and E2F association, yet retains the ability to arrest cells (Kratzke *et al.*, 1994). However, as with E1A, E2F binding in the experiments described here was assayed after incubation of cell lysates at 4°C, and was restricted to analysis of an association with E2F-4. Although we cannot rule out entirely temperature-dependent affinity differences *in vivo*, the clear functional changes observed with tspRb are not accompanied by obvious changes in E1A or E2F association *in vitro*. Thus, it remains possible that temperature-dependent interactions with one or more E2F family members within the cell may be found. Alternatively, tspRb may be defective for transcriptional repression but not E2F association.

We suspect that the temperature dependence of tspRb's ability to repress E2F-dependent transcription may be due to a defect in its ability to interact with the transcriptional machinery adjacent to the E2F-binding sites. It has been proposed that the pRb pocket, after being tethered to a specific promoter through E2F, could either bind surrounding transcription factors, preventing their interaction with the basal transcription machinery (TFIID-like factors; the factor PU.1) (Chow and Dean, 1996), or bind one or more unknown proteins 'X' that have an intrinsic repressor activity (Sellers *et al.*, 1995). Both possibilities would result in the repression of transcription of the affected gene. TspRb may be unable to form such contacts at the non-permissive temperature, but could regain this ability at the permissive temperature. The suppression of proliferation mediated by tspRb at the permissive temperature may thus result from one or more pRb-containing multiprotein complexes that act to repress the transcription of growth-promoting genes. Clearly, our knowledge of pRb's interaction with E2F only scratches the surface of pRb's true molecular role in proliferation control. The tspRb protein promises to be a powerful tool to unravel the molecular mechanism of pRb-mediated transcriptional repression and should also be a very useful tool for identifying genes that are turned on or off soon after temperature shift. Such genes would be good candidates for direct control by pRb. As a first step in the identification of such genes, we recently have established cell lines stably expressing tspRb that undergo growth arrest at the permissive temperature (F.Tiemann and P.W.Hinds, unpublished observations).

The utility of tspRb as a reagent to study pRb's molecular functions in tumor suppression is enhanced by its apparent ability to be inactivated efficiently by temperature upshift. Cell cycle re-entry of tspRb-arrested SAOS-2 cells occurred well within 24 h as determined by BrdU incorporation, suggesting pRb's control of E2F and other factors is rather rapidly reversible. Most interestingly, although these cells displayed a reversible block to S phase entry, they were terminally blocked from proliferation, since they died by apoptosis after shift to the non-permissive temperature. An intriguing property of pRb-expressing SAOS-2 cells is the appearance of the so-

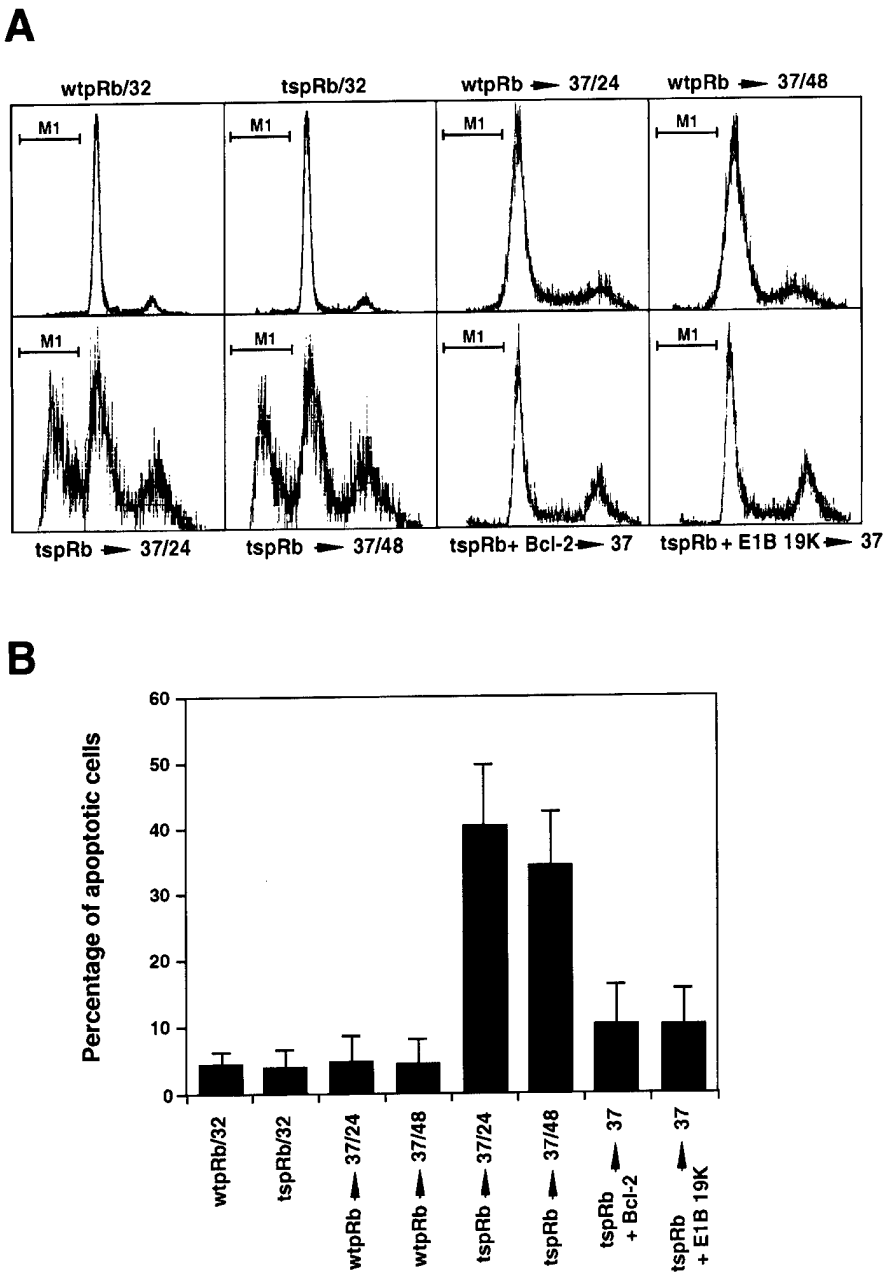

Fig. 8. Inactivation of tspRb after induction of the flat cell phenotype leads to apoptosis. SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668) and 5 μg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7–10 days, when only flat cells appeared on the plate, cells were removed from the drug and either incubated further at 32.5°C or shifted from 32.5 to 37°C. WtpRb- and tspRb-transfected cells that were shifted from 32.5 to 37°C were harvested for flow cytometry analysis 24 and 48 h after temperature shift. All other cells were harvested for flow cytometry analysis 48 h after the former were shifted. Total flat cell populations were analyzed. Apoptosis was measured by the accumulation of cells with a sub-G_1 DNA content in an area indicated as M1. A representative FACS analysis is shown (**A**). The graph (**B**) represents the percentage of cells with sub-G_1 content (apoptotic cells) with mean values derived from three independent experiments.

called flat cell phenotype (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Zhu *et al.*, 1993; Qin *et al.*, 1995). It was suggested that these flat cells resemble the senescent phenotype of primary fibroblasts seen after extended time in culture (Templeton *et al.*, 1991), and that this biological activity of pRb may be a manifestation of a less reversible type of exit from the cell cycle leading to senescence or differentiation (Qin *et al.*, 1995; Weinberg, 1995). Prolonged expression of pRb may produce a 'terminal' pseudo-differentiated or senescent state characterized by the flat cell phenotype that is distinct from pRb–E2F-type cell cycle arrest and which is poorly reversible upon loss of pRb. Indeed, some property of the flat cells themselves, such as cytoskeletal structure or cell shape, may contribute directly to the relative irreversibility of the arrested state. Thus, cellular changes caused by the prolonged expression of functional tspRb appear to preclude rather than restore proliferation (but not cell cycle progression) upon removal of a functional pRb as a result of programed cell death. Interruption of this apoptotic response could be achieved

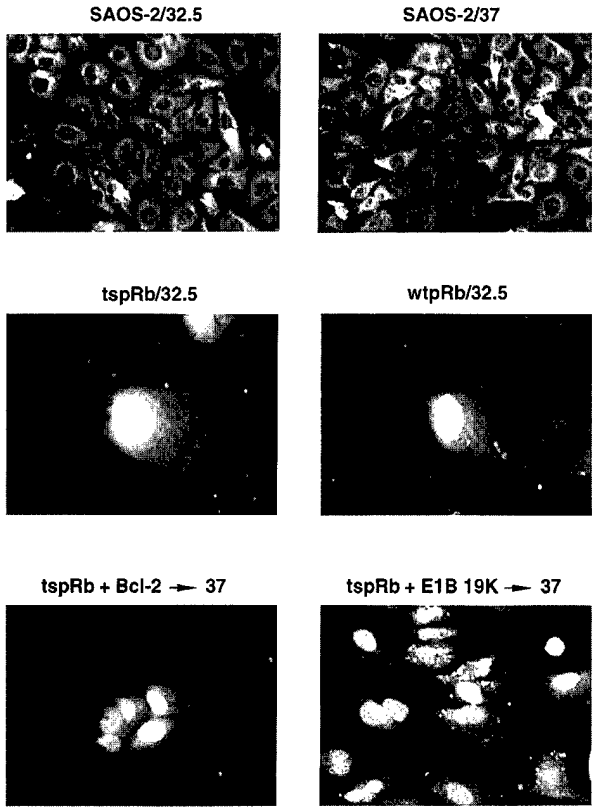

Fig. 9. Inhibition of cell death by apoptosis inhibitors allows the outgrowth of pRb-positive SAOS-2 cells with a normal cell morphology. SAOS-2 cells transfected with tspRb and the apoptosis inhibitors Bcl-2 or E1B 19K, respectively, or with tspRb or wtpRb that were generated at day 7 in the experiment described in Table I, were immunocytochemically stained for pRb and compared with SAOS-2 cells that were cultivated at 37 or 32.5°C. Representive photographs were taken at the same magnification.

by co-transfection of Bcl-2 or E1B 19K, which led to the outgrowth of small, proliferating SAOS-2 cells, positive for the expression of tspRb. As observed by the photographs taken, the outgrowth of colonies was rather slow, with the first appearance of colonies positive for pRb staining at day 7 after temperature shift to the non-permissive temperature. This can be explained in part by the fact that the block to apoptosis was not complete (see Figure 8). Additionally, we cannot rule out a requirement for additional events to restore proliferative capacity to cells that have escaped apoptosis through expression of Bcl-2 or E1B 19K.

Given these results, it is of note that SAOS-2 cells express high levels of the CDK inhibitor gene encoding p16, which is overexpressed in senescent cells, and which is one of the candidate causal senescence genes (Smith and Pereira-Smith, 1996). Since loss of pRb in SAOS-2 cells may allow bypass of the putative p16 function in senescence (Sherr, 1996), re-introduction of a hypophosporylated form of pRb in pRb-negative tumor cells could promote a terminal cell cycle exit by switching on elements of a senescence program. The functional inactivation of pRb once the senescent phenotype is established may lead to apoptosis as a result of two conflicting signals: (i) a growth-promoting signal triggered by a deregulated E2F and (ii) a growth-inhibitory signal maintained by the senescence machinery. In support of this are studies that suggest that several proteins that play a role in regulating cell cycle progression also have an apoptotic potential and that the apoptotic signal induced by such proteins is a direct consequence of conflicting growth control functions. Of particular interest are recent studies that suggest a p53-independent apoptotic activity of E2F-1 that is dependent on the deregulation of a functional pRb–E2F-1 repressor complex. Thus, derepression of genes containing E2F sites may lead directly to apoptosis if the cell's interpretation of its environment is otherwise at odds with a decision to proliferate (Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

In summary, we propose that the introduction of a functional pRb protein into SAOS-2 cells acts as a negative epigenetic signal that is followed by the start of a senescence program (or alternatively a differentiation program) that in turn causes the observed S phase block accompanied by the shape change of the cells. Once the growth-inhibitory signal is established, removal of functional pRb restores S phase entry, but this may conflict with irreversible negative signals resulting from other downstream effects of pRb with the consequent death of the affected cells. Achieved here with a temperature-sensitive pRb, such a transitory reactivation of the pRb pathway has broader conceptual implications for cancer therapy, since techniques designed to restore the pRb pathway only temporarily in tumor cells may prove more efficient at cell killing than permanent expression of a given cell cycle regulator.

Materials and methods

Cell culture, plasmids and transfections

The human osteosarcoma cell line SAOS-2, subclone 2.4 (Hinds *et al.*, 1992) was used for all studies. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 15% heat-inactivated fetal bovine serum in a 3% CO_2 incubator at 37°C.

All pRb expression plasmids were constructed in pSVE (Templeton *et al.*, 1991). The human pRb expression vector phRbc-SVE (here referred to as wtpRb) has been described previously (Templeton *et al.*, 1991; Hinds *et al.*, 1992) and was used in all transfection experiments to express wild-type pRb. Several mutant cDNAs were constructed in this vector by inserting an *Xho*I linker in-frame into different restriction sites at certain codons (Mittnacht *et al.*, 1991; Hinds *et al.*, 1992). HX108 has a 12 bp linker inserted at the *Hin*cII site at codon 108, resulting in the insertion of four amino acids (P-L-E-R) at this position. AcX414 has an insertion of four amino acids (I-P-R-G) at codon 414, resulting from a 10 bp linker inserted into an *Acc*I site. The mutant XX668 carries the 12 bp linker inserted at the *Xmn*I restriction site at codon 668, with four new amino acids (R-S-S-G) at this position. AX763 was constructed by inserting a 12 bp linker into an *Alw*NI site at codon 763, and has three new amino acids (P-L-E) at this position.

The expression vector pSVΔ22 encoding a pRb protein deleted in exon 22 (here referred to as Δ22) was used as a negative control for pRb expression and function. The Δ22 cDNA was derived from the NCI-H592 small cell lung carcinoma (Horowitz *et al.*, 1990) and subcloned into pSVE (Templeton *et al.*, 1991).

The vector pBabepuro (Morgenstern and Land, 1990) was used to mediate resistance to puromycin.

SAOS-2 cells ($1–2\times10^6$) were transfected with the indicated plasmids on 10 cm dishes for 18 h by using the 2× Bes-buffered saline (2× BBS)/calcium phosphate method of Chen and Okayama (1987), with modifications as described previously (Hinds *et al.*, 1992).

Flat cell assay and colony formation assay

For flat cell assays and colony formation assays, cells were transiently transfected with 1.5 μg of pBabepuro and 20 μg of the indicated pRb expression plasmid. Following transfection, cells were plated at 5×10^5 per 10 cm dish. Puromycin (Sigma) was added at 0.5 μg/ml 24 h after plating and cells were selected at either 37 or 32.5°C. After 7–10 days

of selection, cells were stained with crystal violet and flat cells were quantitated as described by Hinds *et al.* (1992). For the quantitation of colonies, cells were stained with crystal violet after 14 days of selection, when macroscopic colonies became detectable. Since no macroscopic colonies were detectable at this time on plates maintained at 32.5°C, microcolonies were quantitated as described for the flat cells.

Flow cytometry analysis

Flow cytometry analysis for the determination of cell cycle profiles of pRb-transfected cells was performed as described previously for pRb and p107 (Zhu *et al.*, 1993). Briefly, 2 μg of the expression plasmid for the B cell surface marker CD20 (pCMVCD20) (van den Heuvel and Harlow, 1993) was co-transfected with the indicated amounts of pRb expression constructs into SAOS-2 cells. At 48 h after the removal of DNA precipitates, cells were rinsed off the plates with phosphate-buffered saline (PBS) containing 0.1% EDTA, pelleted, and stained with 20 μl of a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 monoclonal antibody (PharMingen). Subsequently, cells were fixed with 90% ethanol on ice for several hours. Before flow cytometry analysis, the cells were treated with 200 μg/ml RNase A for 15 min at 37°C and stained with a solution containing 20 μg/ml of propidium iodide. Flow cytometry analysis was performed on a Becton-Dickinson FACScan. The intensity of propidium iodide staining was analyzed with the CellFIT Cell Cycle Analysis software to determine the DNA content and hence the cell cycle profiles on cells that were positive for FITC staining. We observed small variations in the G_1, S and G_2/M populations between samples that were transfected independently with the same plasmids. However, the differences between controls and testing samples observed in each particular experiment were significantly consistent in all separate experiments.

Immunoblotting, immunoprecipitations and in vitro binding assay

The expression of transfected cDNAs was monitored by immunoblotting. Aliquots of SAOS-2 cells, transfected with the indicated amounts of pRb expression constructs for cell cycle analysis, reporter assays or *in vitro* binding assays, were lysed in ELB (50 mM HEPES, pH 7.2; 250 mM NaCl; 2 mM EDTA; 0.1% NP-40) 48 h after the removal of DNA precipitates as described (Latham *et al.*, 1996). Protein concentrations in the cell lysates were determined by the Bio-Rad protein assay. Proteins were separated by SDS–PAGE and transferred to nitrocellulose by standard procedures. pRb proteins were monitored by blotting with the monoclonal antibodies 245 (PharMingen) or Ab-5 (Oncogene Science). Detection was performed, after incubation with peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch), by enhanced chemiluminescence (Amersham), as described previously (Latham *et al.*, 1996).

For E2F-4 immunoprecipitations, lysates containing 200 μg of protein were subjected to immunoprecipitation with 100 μl of hybridoma supernatant from the monoclonal antibody 2-4/E9 (kind gift from J.Lees), as described (Latham *et al.*, 1996).

Plasmids encoding the GST–E1A$_{12S}$ and E1A$_{12S}$ pm928/961 fusion proteins (Kraus *et al.*, 1994) were used to produce the proteins for the GST pull down experiments. GST fusion proteins were induced, purified and recovered on glutathione–Sepharose beads as described earlier (Kaelin *et al.*, 1991). The beads were rocked with aliquots of cell lysates for 2 h at 4°C and then washed five times with ELB. The beads were then boiled in sample buffer and bound proteins were resolved by SDS–PAGE. pRb proteins were visualized by immunoblotting as described above. With each aliquot of cell lysate, ~150 μg of total protein was analyzed.

Nuclear extraction

SAOS-2 cells were transfected with 20 μg of the indicated pRb expression construct and incubated at either 37 or 32.5°C. At 48 h after the removal of DNA precipitates, transfected cells were subjected to subcellular fractionation as described previously (Mittnacht and Weinberg, 1991). Briefly, cells were lysed in a hypotonic buffer (10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol) containing 0.1% Triton X-100, and fractionated by low-speed centrifugation into a low-salt-soluble supernatant and an insoluble nuclear pellet. The insoluble nuclear pellet fraction then was extracted further with ELB. pRb in aliquots of both extracts was detected by immunoblotting as described above. To control for pRb expression, a small aliquot of each sample was lysed directly in ELB and monitored for pRb expression by immunoblotting.

E2F transcription assay

Luciferase assays using the luciferase reporter plasmid pGL2-AN, containing the E2F-1 promoter upstream of the luciferase cDNA (Neuman *et al.*, 1994), were performed according to standard protocols (Ausubel *et al.*, 1990). Cells were co-transfected with 5 μg of pGL2-AN, 2 μg of pCMV-βgal and the indicated amount of different pRb expression plasmids, and incubated at 37 or 32.5°C. At 48 h after the removal of DNA precipitates, β-galactosidase and luciferase activities were assayed.

Analyses of DNA synthesis and cell death, and immunocytochemical staining for pRb

SAOS-2 cells were transfected with 1.5 μg of pBabepuro, 20 μg of the indicated pRb expression plasmid and 5 μg of either the Bcl-2 expression vector SFFV-Bcl-2 (kindly provided by Dr S.Korsmeyer) or the E1B 19K expression vector pCMV-E1B 19K (Han *et al.*, 1996), when indicated. Following transfection, cells were plated and selected at 32.5°C as described for the flat cell assays. After 7–10 days, when only flat cells appeared on the plates, cells were removed from the drug and either incubated further at 32.5°C or shifted to 37°C. Treated cells as well as control cell populations were then scored for (i) DNA synthesis by measuring their ability to incorporate BrdU, (ii) cell death by cell number comparison and flow cytometry analysis of propidium iodide-labeled cells and (iii) pRb expression by immunocytochemical staining for pRb.

(i) BrdU incorporation was detected exactly as described earlier (Latham *et al.*, 1996). At least 100 nuclei per sample were counted.

(ii) To compare cell numbers, photographs of cells within a fixed area of 0.25 cm^2 were taken on seven consecutive days and the cells in this fixed area were counted. Relative cell numbers based on several experiments are presented. Flow cytometry analysis was carried out and total flat cell populations were gated and analyzed as described (Phillips *et al.*, 1997).

(iii) The immunocytochemical staining for pRb was performed as described (Latham *et al.*, 1996).

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